Antioxidant activity of hydroalcoholic extract of *Ferula gummosa* Boiss roots

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Abstract. – Objectives: *Ferula gummosa* Boiss is native to central Asia. This plant has traditionally been used in the treatment of many diseases. The antihypoxic and antioxidant activities of *Ferula gummosa* roots were investigated.

Material and Methods: 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), nitric oxide and hydrogen peroxide scavenging activities, Fe²⁺ chelating ability, reducing power and hemoglobin-induced linoleic acid peroxidation were used to evaluate antioxidant activities. Antihemolytic activity was evaluated by H₂O₂ induced hemolysis in rat erythrocytes. The total amount of phenolic compounds was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve.

Results: The extracts showed moderate antioxidant activity in some models. IC⁵₀ for DPPH radical-scavenging activity was 579.6±19.4 µg/ml. The extracts showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg ml⁻¹ but showed good Fe²⁺ chelating ability. IC⁵₀ was 895.5±24.1 µg/ml. The extract also exhibited low antioxidant activity in the linoleic acid model but were capable of scavenging hydrogen peroxide in a concentration dependent manner. Tested extract showed moderate activity in H₂O₂ induced hemolysis in rat erythrocytes which was not comparable with vitamin C.

Conclusions: *F. gummosa* Boiss root showed different level antioxidant and antihemolytic activities. Biological effects may be attributed, at least in part, to the presence of phenols and flavonoids in the extract.

Key Words:

Introduction

Free radicals are usually short-lived species but they possess a single unpaired electron, rendering them highly reactive against biologically important macromolecules including DNA, proteins and membrane lipids. To counteract this threat to their integrity, cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidant species, and on antioxidant enzymes. A high proportion of the antioxidant systems of the human body are dependent on dietary constituents¹. Synthetic antioxidants such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) are used to decelerate these processes. However, due to their unstable and highly volatile nature, they have frequently brought some questions about their safety and efficiency ever since their first introduction to the food industry². Consequently, the need to identify alternative natural and safe sources of antioxidant arose and the search for safe and natural antioxidants, especially of plant origin, has notably increased in recent years³. *Ferula (F.)* gummosa Boiss. (*Apiaceae*) is a perennial plant native to central Asia, growing in the northern and western parts of Iran and blooms once in its several years’ life⁴. Nomads of southwest Iran call this plant “Barijeh” and traditionally use its resin for the treatment of diarrhea. They eat a small piece of the resin and believe it to be a very effective anti-diarrheal herbal medicin⁵. In Iranian ancient medicine, the gum obtained from the aerial parts of this plant has been used for stomach pain, chorea, epilepsy and as a wound-healing remedy⁶⁷. In recent years there are some reports regarding the central effects of this plant.
An antinociceptive activity has been shown for the hydroalcoholic extract of aerial parts and acetone extract of *F. gummosa* seed and root were reported previously. Furthermore, a methanol-chloroform (1:1) extract of *F. gummosa* and its fractions have alleviated the morphine withdrawal syndrome induced by naloxone. Anticonvulsant potential of an essential oil and antibacterial activity of the seed and root of *F. gummosa* reported previously. Composition of the essential oil of the fruit of the plant has been determined. We recently have reported good antioxidant activity of *Ferula assafoetida* and *F. gummosa* leaves, flowers or stems. To best of our knowledge there is no scientific report on antioxidant activity of *F. gummosa* roots, so the aim of this study was to determine the antioxidant and anti-hemolytic activities of hydroalcoholic extract of *F. gummosa* root in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

### Materials and Methods

#### Chemicals

Trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide and hydrogen peroxide H2O2 were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA) and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

#### Plant Materials and Preparation of Freeze-dried Extract

*F. gummosa Boiss* root was collected from Gadouk area, south of Ghaemshahr, Iran, in 2009 and identified by Dr. Bahman Eslami (Assistance Professor of Plant Systematic, Islamic Azad University, Ghaemshahr, Iran). Voucher specimens are deposited with the Faculty of Pharmacy Herbarium (No. GRF 32). Sample was dried at room temperature and coarsely ground before extraction. A known amount of sample was extracted at room temperature by percolation method using ethanol/water (70:30). The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

#### Determination of Total Phenolic Compounds and Flavonoid Contents

Total phenolic compound contents were determined by the Folin-Ciocalteau method. The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Result was expressed as gallic acid equivalents. Total flavonoids were estimated as previously described. Briefly, 0.5 ml solution of extract in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (UV-Visible EZ201, Perkin Elmer, Norwalk, CA, USA). Total flavonoid contents were calculated as quercetin from a calibration curve.

#### Antioxidant Activity

##### DPPH Radical-Scavenging Activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the extracts. Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC50 values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

##### Determination of Metal Chelating Activity

The ability of the *F. gummosa Boiss* root extract to chelate ferrous ions was estimated by our recently published papers. Briefly, different concentrations of extract were added to a solution of 2 mM FeCl2 (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixtures were then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solutions was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe2+ complex forma-
tion was calculated as \[\frac{(A_0 - A_1)}{A_0} \times 100\], where \(A_0\) was the absorbance of the control, and \(A_1\) of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

**Assay of Nitric Oxide-Scavenging Activity**

The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of extract dissolved in water and incubated at room temperature for 150 min. The same reaction mixture without the extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control\(^{12}\).

**Scavenging of Hydrogen Peroxide**

Briefly, a solution of \(\text{H}_2\text{O}_2\) (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of \(\text{H}_2\text{O}_2\) was determined by absorption at 230 nm using a spectrophotometer (UV-Visible EZ201, Perkin Elmer, Norwalk, CA, USA). Extract (0.1-3.2 mg/ml) in distilled water was added to a \(\text{H}_2\text{O}_2\) solution (0.6 ml, 40 mM). The absorbance of \(\text{H}_2\text{O}_2\) at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without \(\text{H}_2\text{O}_2\). The percentage of \(\text{H}_2\text{O}_2\) scavenging by the extracts and standard compounds was calculated as follows: % Scavenged \[\frac{[\text{H}_2\text{O}_2]}{[\text{H}_2\text{O}_2]} = \frac{(A_0 - A_1)}{A_0} \times 100\] where \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance in the presence of the sample of extract and standard\(^{16}\).

**Reducing Power Determination**

Briefly, 2.5 ml of extract (25-800 mg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide \([\text{K}_3\text{Fe(CN)}_6]\) (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl\(_3\) (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control\(^{15}\).

**Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test**

The antioxidant activity of extract was determined by a modified photometry assay\(^{19}\). Reaction mixtures (200 ml) containing 10 ml of each extract (10-400 mg), 1 mmol/l of linoleic acid emulsion, 40 mmol/l of phosphate buffer (pH 6.5) and 0.0016% hemoglobin were incubated at 37°C for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480 nm (UV-Visible EZ201, Perkin Elmer, Norwalk, CA, USA) after coloring with 100 ml of 0.02 mol/l of FeCl\(_2\) and 50 ml of ammonium thiocyanate (30 g/100 ml). Vitamin C was used as positive control.

**Antihemolytic Activity**

**Preparation of Rat Erythrocytes**

All the animal experiments were carried out with the approval of institutional animal Ethical Committee. Male Wistar rats in the body weight range of 180-220 g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were sacrificed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan et al\(^{19}\). Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4°C, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4°C and used within 6 h for further studies.

**Antihemolytic Activity of Extracts Against \(\text{H}_2\text{O}_2\) Induced Hemolysis**

The inhibition of rat erythrocyte hemolysis by the extract was evaluated according to our recently published paper\(^{20}\). The rat erythrocyte hemoly-
sis was performed with H\textsubscript{2}O\textsubscript{2} as free radical initiator. To 100 µl of 5% (v/v) suspension of erythrocytes in PBS, 50 µl of each extracts with different concentrations (5-25 µg in PBS pH 7.4), which corresponds to 100-3200 µg of extract, was added. To this, 100 µl of 100 IM H\textsubscript{2}O\textsubscript{2} (in PBS pH 7.4) was added. The reaction mixtures were shaken gently while being incubated at 37°C for 3 h. The reaction mixtures were diluted with 8 ml of PBS and centrifuged at 2000×g for 10 min. The absorbances of the resulting supernatants were measured at 540 nm by spectrophotometer (UV-Visible EZ201, Perkin Elmer, Norwalk, CA, USA) to determine the hemolysis. Likewise, the erythrocytes were treated with 100 µM H\textsubscript{2}O\textsubscript{2} and without inhibitor (plant extract) to obtain a complete hemolysis. The absorbance of the supernatants was measured at the same condition. The inhibitory effect of the extract was compared with standard antioxidant vitamin C. To evaluate the hemolysis induced by extract, erythrocytes were preincubated with 50 µl of extracts corresponding to 25 µg extracts for 1 h and the hemolysis was determined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 µM H\textsubscript{2}O\textsubscript{2} as 100%. The IC\textsubscript{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

**Statistical Analysis**

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance (p < 0.05) and the means separated by Duncan’s multiple range tests. The EC\textsubscript{50} values were calculated from linear regression analysis.

**Results and Discussion**

Total phenol compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve (y = 0.00054x + 0.0628, r\textsuperscript{2} = 0.987). The total phenolic contents of extract were 46.3 ± 1.7 mg gallic acid equivalent/g of extract. The total flavonoid contents were 15.47 ± 0.5 mg quercetin equivalent/g of extract powder, by reference to standard curve (y = 0.0063x, r\textsuperscript{2} = 0.999). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities\textsuperscript{21}. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron-donation. Substances which are able to perform this reaction can be considered as antioxidants and, therefore, radical scavengers\textsuperscript{22}. IC\textsubscript{50} for DPPH radical-scavenging activity was 579.6 ± 19.4 µg/ml. The IC\textsubscript{50} values for ascorbic acid, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1 mg/ml, respectively. Phenol and flavonoid contents of this plant may lead to its good DPPH-scavenging activity\textsuperscript{23}. Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as thalassemia major\textsuperscript{23}. In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer’s disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD\textsuperscript{24}. Foods are often contaminated with transition metal ions which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry\textsuperscript{25}. These processes can be delayed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease\textsuperscript{18}. Therefore, minimizing Fe\textsuperscript{2+} concentration affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated according to our recent papers\textsuperscript{17,18}. Ferrozine can quantitatively form complexes with Fe\textsuperscript{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The extract showed good Fe\textsuperscript{2+} chelating ability. IC\textsubscript{50} was 895.5 ± 24.1 µg/ml. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion\textsuperscript{24}. The nitric oxide assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH
spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The % inhibition was increased with increasing concentration of the extracts. The extract showed weak nitric oxide-scavenging activity (only 30% at 1600 µg ml⁻¹ vs. quercetin with IC₅₀ = 20 ± 0.01 µg/ml). Although quercetin showed very potent NO radical scavenging, its carcinogenic activity has been reported. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. Scavenging of H₂O₂ by F. gummosa extract may be attributed to their phenolics, and other active components which can donate electrons to H₂O₂, thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. Extract showed good activity (IC₅₀ = 394.1 ± mg/ml). The IC₅₀ values for vitamin C and BHA were 21.4 ± 1.1 and 52 ± 2.6 mg/ml, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H₂O₂ is very important throughout food systems. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl’s Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose response curves for the reducing powers of extract. There was significant difference between extract and vitamin C (p < 0.01). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and, therefore, should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts. Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O₂ transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O₂ species. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radicals. Hydroxyl radi-

![Figure 1](image-url)
Antioxidant activity of hydroalcoholic extract of *Ferula gummosa* Boiss roots

Antioxidants eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested extract show moderate activity in hemoglobin-induced linoleic acid system, but it was not comparable with vitamin C ($p < 0.001$) (Figure 2). The effect of *F. gummosa* root extract was tested and found that they did not show any harmful effects on erythrocytes (IC$_{50}$ was 845.6±58.3 µg/ml vs. vitamin C 235±9.1 µg/ml). Anti-hemolytic activity of quercetin and other flavonoid previously reported and good activity of extracts maybe result of high flavonoid content especially quercetin$^{30}$.

**Conclusion**

Our studies indicate that the hydroalcoholic extract of *F. gummosa* Boiss root showed different level antioxidant and antihemolytic effect those maybe results of high phenol and flavonoid content. It is, therefore, very promising for further biochemical experiments, which will be focused on evaluating the mechanism of this activity.

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**References**


